

# Cold Calcium Signaling in Arabidopsis Involves Two Cellular Pools and a Change in Calcium Signature after Acclimation

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Cold shock elicits an immediate rise in cytosolic free calcium concentration ( $[Ca^{2+}]_{cyt}$ ) in both chilling-resistant *Arabidopsis* and chilling-sensitive tobacco (*Nicotiana glauca*). In *Arabidopsis*, lanthanum or EGTA caused a partial inhibition of both cold shock  $[Ca^{2+}]_{cyt}$  elevation and cold-dependent *kin1* gene expression. This suggested that calcium influx plays a major role in the cold shock  $[Ca^{2+}]_{cyt}$  response and that an intracellular calcium source also might be involved. To investigate whether the vacuole (the major intracellular calcium store in plants) is involved, we targeted the calcium-dependent photoprotein aequorin to the cytosolic face of the vacuolar membrane. Cold shock calcium kinetics in this microdomain were consistent with a cold-induced vacuolar release of calcium. Treatment with neomycin or lithium, which interferes with phosphoinositide cycling, resulted in cold shock  $[Ca^{2+}]_{cyt}$  kinetics consistent with the involvement of inositol trisphosphate and inositol phosphate signaling in this response. We also investigated the effects of repeated and prolonged low temperature on cold shock  $[Ca^{2+}]_{cyt}$ . Differences were observed between the response of *Arabidopsis* and *N. glauca* to repeated cold stimulation. Acclimation of *Arabidopsis* by pretreatment with cold or hydrogen peroxide caused a modified calcium signature to subsequent cold shock. This suggests that acclimation involves modification of plant calcium signaling to provide a “cold memory.”

## INTRODUCTION

Calcium is a second messenger used in many plant signaling processes (Gilroy et al., 1990; Knight et al., 1991; Shacklock et al., 1992; Franklin-Tong et al., 1993; Bush, 1995). We have shown previously that low-temperature signaling is one such process and that cold shock in tobacco is accompanied by a large transient rise in cytosolic calcium concentration ( $[Ca^{2+}]_{cyt}$ ) (Knight et al., 1991). It has been speculated previously that cold damage in plants may be due to a lack of calcium homeostasis and subsequent calcium toxicity (Minorsky, 1985), but more recent data suggest that a change in calcium levels is a necessary step in a temperature-sensing mechanism that enables the plant to withstand future cold stress better (Minorsky, 1989; Minorsky and Spanswick, 1989). Brief rises in  $[Ca^{2+}]_{cyt}$  are one of a number of transient physiological responses that have been observed during rapid cooling to nonfreezing temperatures (chilling), including effects on protoplasmic streaming, growth, phloem translocation, water absorption, membrane potential, and cell motility (Minorsky, 1989). Chilling-sensitive plants characteristically exhibit structural injuries and may suffer from metabolic dysfunction when chilled, whereas chilling-resistant plants show improved performance of cells at low temperatures and an increased resistance to freezing (Kacperska, 1989).

The acquisition of this freezing tolerance by exposure to non-freezing, noninjurious low temperatures is known as cold acclimation (Guy, 1990). Acclimation causes accumulation of cryoprotectants, including polyamines, changes in enzyme activities, physical and biochemical restructuring of cell membranes through changes in the lipid components (Lynch and Steponkus, 1987), and induction of other nonenzymatic proteins that alter the freezing of water (Guy, 1990). It has been suggested that the proteins encoded by the cold-induced *Arabidopsis* genes *kin1* and *kin2* may act in such a way because they are similar in sequence to the fish “antifreeze” proteins that have this function (Kurkela and Franck, 1990; Kurkela and Borg-Franck, 1992). Calcium signaling has been implicated in cold acclimation in chill-resistant plants (Monroy et al., 1993; Monroy and Dhindsa, 1995).

Transgenic *Arabidopsis* and tobacco expressing the calcium-sensitive photoprotein aequorin have been used successfully to report changes in intracellular calcium levels in response to a number of signals by using either luminometry of whole plants (Knight et al., 1991, 1992, 1995; Knight and Knight, 1995) or luminescence imaging of whole plants and specific tissues (Knight et al., 1993). However, the levels of luminescence produced during signaling are too low for imaging on a subcellular level to be possible. To overcome this limitation, recent work has further refined the recombinant aequorin technique by

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using topogenic sequences targeting the protein to specific subcellular sites. By directing aequorin expression to a specific subcellular location, simple luminometry reveals calcium changes occurring at that particular site in the cell. Aequorin has been successfully targeted to the nucleus (Brini et al., 1993), mitochondrion (Rizzuto et al., 1992; Badminton et al., 1995), and endoplasmic reticulum (ER; Kendall et al., 1992) of mammalian cells as well as the chloroplasts (Johnson et al., 1995) and ER (N.J. Watkins, H. Knight, J.M. Kendall, A.K. Campbell, A.J. Trewavas, and M.R. Knight, unpublished results) of plants, allowing free calcium measurements to be made at these subcellular locations.

Key questions arise regarding the role of calcium in vivo during low-temperature signaling and acclimation. Previous studies have shown that lanthanum does not completely inhibit calcium-induced luminescence following cold shock; whether extracellular calcium can therefore be solely responsible for cold-induced  $[Ca^{2+}]_{cyt}$  elevation or whether there is a role for intracellular stores remains to be determined. What mechanisms control the release of calcium from these stores? Do cold-resistant and cold-sensitive plants undergo different calcium signaling in response to cold? Is low-temperature calcium signaling modified as a result of cold acclimation in cold-resistant plants?

## RESULTS

### Cold Shock $[Ca^{2+}]_{cyt}$ Response and Inhibition of Calcium Influx by EGTA and Lanthanum

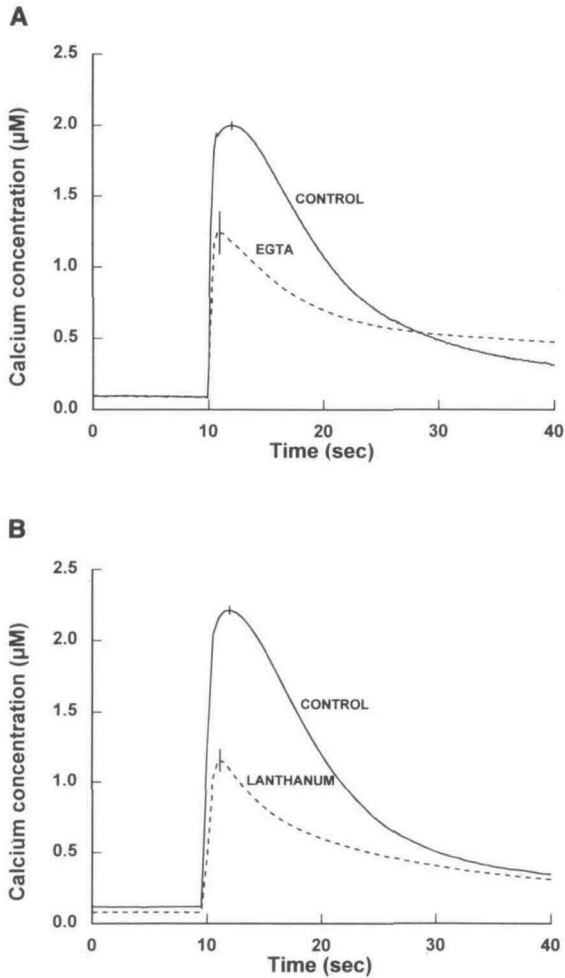
Due to previous difficulties encountered with the calibration of free calcium concentrations measured by using aequorin, previous data have been presented simply as changes in luminescence. However, we are now able to present our results as calibrated free calcium concentrations by using a new calibration specific for the isoform of aequorin and temperature that we used in these experiments. Because the relationship between calcium levels and luminescence is double logarithmic (Campbell, 1988), the low levels of calcium-dependent luminescence seen during cold shock in lanthanum-treated plants (Knight et al., 1991) gave a disproportionately high estimate of the extent of inhibition. This demonstrates the vital importance of using calibrated  $[Ca^{2+}]_{cyt}$  data for the interpretation of results.

We tested whether cold shock mediates  $[Ca^{2+}]_{cyt}$  changes in Arabidopsis that are similar to those in tobacco and the extent to which EGTA and lanthanum inhibit these changes. As shown in Figure 1, cold shock treatment of 7-day-old Arabidopsis seedlings expressing cytosolic aequorin immediately stimulated a peak of  $[Ca^{2+}]_{cyt}$  of  $2.2 \pm 0.034 \mu M$  ( $n = 6$ ). Data presented for  $[Ca^{2+}]_{cyt}$  in this study are all averages of replicate traces from individual seedlings, which showed a high level of consistency. Preincubating seedlings with either EGTA or lanthanum (III) chloride reduced the magnitude of the cold re-

sponse (Figure 1), confirming earlier data suggesting that the response involves an influx of  $Ca^{2+}$  (Knight et al., 1991; Monroy and Dhindsa, 1995). This reduction was not complete, contrary to earlier indications in tobacco (Knight et al., 1991). However, proper calibration of tobacco cold shock  $[Ca^{2+}]_{cyt}$  showed similar results (data not shown). The cold shock  $Ca^{2+}$  peak in the cytosol was considerably reduced, to  $1.3 \pm 0.17 \mu M$  ( $n = 4$ ) after preincubation in 20 mM EGTA for 1 hr (Figure 1A). The later part of the response was prolonged by EGTA, with  $[Ca^{2+}]_{cyt}$  remaining at significantly higher levels than controls for at least 40 sec after stimulation. EGTA is not thought to enter cells but chelates calcium in the cell wall, making it unavailable for entry through the plasma membrane into the cytosol. The effect of a 1-hr incubation in EGTA is probably to lower gradually the level of  $Ca^{2+}$  in the cytosol, which is subsequently replenished by the intracellular stores. With this depletion of intracellular  $Ca^{2+}$ , calcium-stimulated  $Ca^{2+}$  pumps on the membranes (Evans, 1994) may work less efficiently and against smaller gradients and may take longer to remove the raised levels of  $Ca^{2+}$  following stimulation.

After 6 hr of pretreatment in 10 mM lanthanum chloride, the cold shock response was significantly reduced, with peak height approximately half ( $1.2 \pm 0.092 \mu M$ ;  $n = 6$ ) of control levels (Figure 1B). Even though there is evidence that lanthanides can enter plant cells (Quiquampoix et al., 1990) and intracellular channels are undoubtedly inhibited by lanthanum (Gelli and Blumwald, 1993; Allen and Sanders, 1994; Klusener et al., 1995), the similarity between the results obtained with lanthanum and EGTA suggests that lanthanum is acting on plasma membrane calcium channels and is preventing an influx of calcium. These data again suggest that entry of extracellular  $Ca^{2+}$  into the cytosol is responsible for a substantial part of the  $[Ca^{2+}]_{cyt}$  cold shock response. However, the lack of complete inhibition suggests that other intracellular stores may be involved. The possibilities of intracellular  $Ca^{2+}$  stores being involved in cold signaling have been overlooked because previous studies measured only calcium uptake (Monroy and Dhindsa, 1995) or used uncalibrated aequorin measurements (Knight et al., 1991).

Expression of the cold- and abscisic acid-inducible gene *kin1* (Kurkela and Franck, 1990) was used as a marker for an end response after cold shock treatments. Total RNA was extracted 1 hr (a time period determined by an earlier time course experiment; data not shown) after cold shock treatment of Arabidopsis plants expressing cytosolic aequorin, after which cDNA was synthesized. *kin1* cDNA was selectively amplified by polymerase chain reaction (PCR), using specially designed primers under conditions in which the amplification reaction was exponential. The primers were designed to amplify the whole of the coding sequence, and the reverse primer was chosen based on its homology with the 3' flanking sequence of *kin1*, which differs from that of *kin2*, a second gene from the same family, but a gene that is induced by drought and salinity stress as well as cold (Kurkela and Borg-Franck, 1992). These primers also were designed to span an intron so as to be able to discriminate between the amplification of bona fide



**Figure 1.** Effect of Inhibition of Calcium Influx on Elevation of  $[Ca^{2+}]_{cyt}$  by Cold Shock and on *kin1* Expression after Cold Shock.

**(A)** Effect of preincubation for 1 hr in 20 mM EGTA (dashed line) on cold shock-induced  $[Ca^{2+}]_{cyt}$  elevation compared with the control (solid line).

**(B)** Effect of preincubation for 6 hr in 10 mM lanthanum chloride (dashed line) on cold shock-induced  $[Ca^{2+}]_{cyt}$  elevation compared with the control (solid line).

Traces shown are averages of four **(A)** or five **(B)** individual seedlings. Vertical lines on peaks represent  $\pm$  SE.

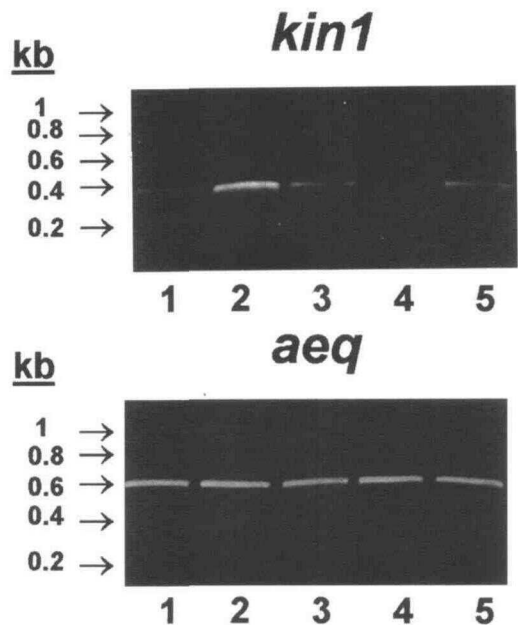
transcripts from that of possible contamination with genomic DNA. These larger amplification products were never detected in these experiments.

A single cold shock (Figures 1A and 1B) caused a significant induction of expression of the cold-induced gene *kin1* in cytosolic aequorin plants (Figure 2). Expression was substantially, but only partially, reduced when the cold treatment was preceded by a 1-hr treatment in 10 mM lanthanum chloride. EGTA treatment also caused a reduction in *kin1* expression but less than with lanthanum. This indicates that the induction of this cold-regulated gene occurs via an influx of  $Ca^{2+}$ ,

and if the  $Ca^{2+}$  influx is prevented, the gene is expressed to a lesser extent. This partial inhibition of gene expression also has been observed with the cold acclimation-specific (*cas*) genes of alfalfa (Monroy et al., 1993). In this case, concentrations of  $La^{3+}$  that were able to inhibit  $Ca^{2+}$ -dependent cold acclimation in cell suspensions were not able to inhibit *cas* expression fully. Incubation of plants for 1 hr with 100 mM calcium chloride to cause an elevation in  $[Ca^{2+}]_{cyt}$  (McAinsh et al., 1995) without a subsequent cold shock also caused some induction of *kin1* expression but not to the same levels as that seen after cold treatment (Figure 2). This concentration of external calcium caused a transient of  $\sim 1 \mu M$  in our seedlings (data not shown).

#### Determination of Subcellular Location and Activity of Pyrophosphate-Aequorin Fusion Protein

The incomplete inhibition of the cold shock  $[Ca^{2+}]_{cyt}$  elevation by high concentrations of lanthanum and EGTA suggested that,

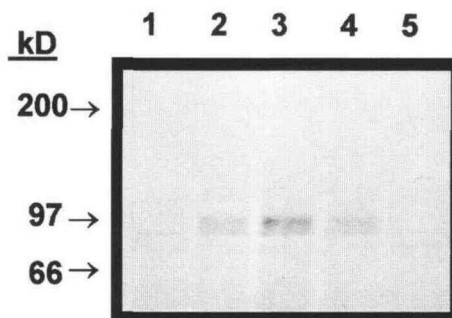


**Figure 2.** Reverse Transcription PCR of *kin1* Transcripts in 7-Day-Old Arabidopsis seedlings.

Lanes 1 contains the PCR product from tissue incubated in water for 1 hr; lanes 2, tissue incubated in water for 1 hr before cold shock treatment; lanes 3, tissue incubated in 100 mM  $CaCl_2$  for 1 hr; lanes 4, tissue incubated in 10 mM  $LaCl_3$  for 1 hr before cold shock treatment; and lanes 5, tissue incubated in 20 mM EGTA for 1 hr before cold shock treatment. Tissue was collected 1 hr after cold shock stimulation, and RNA was extracted as described in the text. cDNA was synthesized from total RNA, and *kin1* DNA was amplified by PCR. Also shown is reverse transcription PCR of apoaquorin (*aeq*) transcripts from the same tissue. The arrows at left show the positions of the 0.2-, 0.4-, 0.6-, 0.8-, and 1.0-kb markers.

in addition to the influx of extracellular  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  may be supplied to the cytosol by an intracellular store during cold shock. We have targeted aequorin to the ER of *Arabidopsis* by using the presequence of human calreticulin and a "KDEL" for retention (N.J. Watkins, H. Knight, J.M. Kendall, A.K. Campbell, A.J. Trewavas, and M.R. Knight, unpublished data). These plants show high basal levels of luminescence, indicative of the high free calcium concentration of the ER, which increases even further in response to cold shock. This indicates that cold shock does not cause release of calcium from the ER but that the ER takes up calcium from the cytosol after cold shock. Similarly, targeting aequorin to the nucleus by fusion to a nuclear-located *Xenopus* protein, nucleoplasmin (Badminton et al., 1995), shows that there is no nuclear free calcium elevation in response to cold shock in tobacco (A. van der Luit, A. Haley, M.R. Knight, and A.J. Trewavas, unpublished data). It therefore appeared possible that the vacuole, a much named plant  $\text{Ca}^{2+}$  store (Gelli and Blumwald, 1993), could be involved. For this reason, we chose to measure  $[\text{Ca}^{2+}]_{\text{cyt}}$  specifically in the cytosolic microdomain adjacent to the vacuolar membrane ( $[\text{Ca}^{2+}]_{\text{md}}$ ). To facilitate these measurements, *Arabidopsis* was transformed with the chimeric construct *HVA1*, which encodes a proton pyrophosphatase–apoequorin fusion protein. Figure 3 shows a hydrophilicity plot (Kyte and Doolittle, 1982) of the predicted *HVA1* fusion protein. This shows the 13 putative membrane-spanning domains of the pyrophosphatase (Sarafian et al., 1992) and the strongly hydrophilic apoequorin moiety. Putative transformants selected on the basis of kanamycin resistance were screened for aequorin activity in vitro. The highest expressing lines, 2.1, 2.2, and 3.1, were tested for expression of the full-length fusion protein by protein gel blotting.

Protein gel blotting of protein extracts from 3-week-old plants revealed a protein of  $\sim 100$  kD when challenged with a polyclonal anti-aequorin antibody in each of the three independent transformed lines expressing the pyrophosphatase–apoequorin fusion protein (Figure 4). This indicated that the fusion was being expressed (its predicted molecular mass is 103.7 kD)

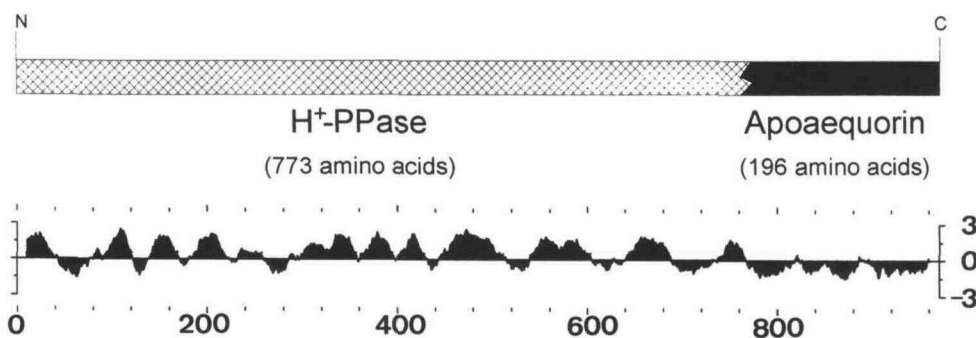


**Figure 4.** Immunoblot of 3-Week-Old *Arabidopsis* Tissue Challenged with an Anti-Aequorin Polyclonal Antibody.

Lane 1 contains seedlings expressing cytosolic aequorin; lane 2, *HVA1* line 3.1; lane 3, *HVA1* line 2.2; lane 4, *HVA1* line 2.1; and lane 5, the wild type. The arrows indicate the position of molecular size markers.

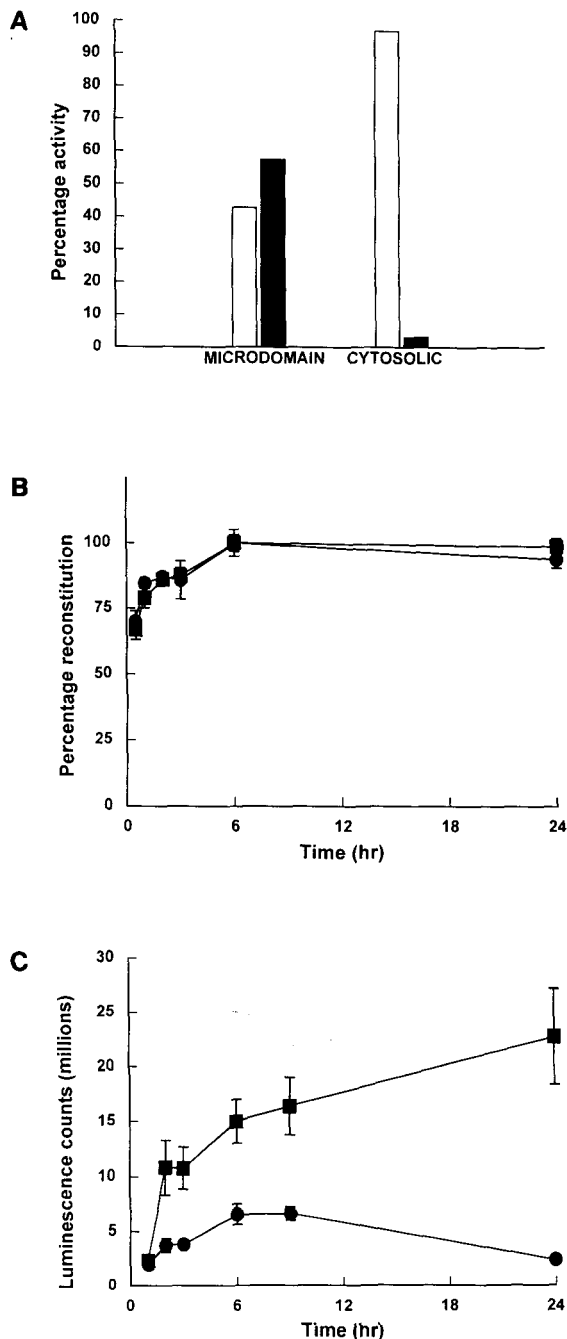
in the transformed lines. This protein was not detected in either wild-type *Arabidopsis* or plants expressing cytosolic aequorin (Figure 4). Because all three lines contained the fusion protein, line 2.2 was selected for further study because both protein gel blot analysis (Figure 4) and in vitro reconstitution experiments (data not shown) indicated that this transgenic line expressed the fusion protein to the highest level.

Centrifugal fractionation of cytosolic targeted aequorin plants and *HVA1*-2.2 plants revealed that 57% of total reconstituted aequorin activity was associated with the 80,000g pellet in *HVA1*-2.2 plants compared with 3% in cytosolic aequorin plants, as shown in Figure 5A. The 80,000g pellet should contain microsomal, vacuolar membranes/vesicles, and plasma membrane; the cytosolic fraction would be expected to be predominantly in the supernatant. To determine further the localization of the pyrophosphatase–aequorin fusion protein in *Arabidopsis*, we adopted the approach employed previously to determine the localization of a fusion protein targeted to the



**Figure 3.** *HVA1* Chimeric Construct.

Shown is a diagram of the predicted pyrophosphatase–apoequorin fusion ( $\text{H}^+$ -PPase). The hydrophobicity plot was calculated according to Kyte and Doolittle (1982), using the PEPLOT program from the Genetics Computer Group (Madison, WI) with a running window of 20 amino acid residues. Hydrophobicity is shown as negative and hydrophilicity as positive. N, N terminus; C, C terminus.



**Figure 5.** Subcellular Fractionation and Aequorin Reconstitution.

(A) The percentage of aequorin activity associated with the soluble and microsomal fractions from cytosolic and microdomain aequorin-expressing Arabidopsis is shown.

(B) In vitro reconstitution time course of extracts from microdomain (■) and cytosolic (●) Arabidopsis plants ( $n = 3$ ).

(C) In vivo reconstitution time course in microdomain (●) and cytosolic (■) Arabidopsis plants ( $n = 3$ ).

Vertical lines on each symbol (in [B] and [C]) indicate  $\pm$  SE.

tonoplast in transgenic tobacco (Höfte and Chrispeels, 1992). This enabled us to calculate the amount of aequorin activity associated with total protoplast and purified vacuole fractions containing the same amount of acid phosphatase activity (as a vacuolar marker). A substantial amount (24%) of aequorin activity resided in the vacuolar fraction after the vacuolar isolation procedure. This activity represented tonoplast-located aequorin facing the cytosol, because conditions inside the vacuole would irreversibly inactivate any aequorin present on the vacuolar side of the tonoplast membrane (Campbell, 1988).

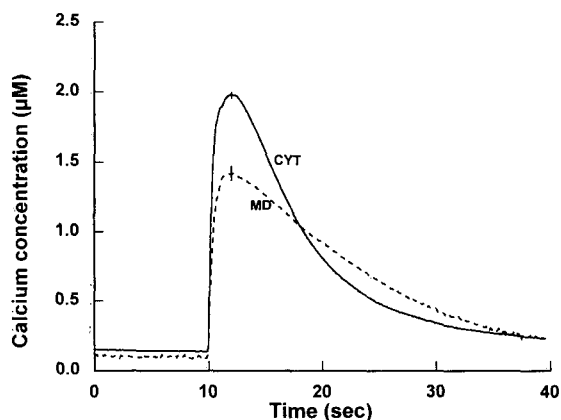
The kinetics of in vivo and in vitro reconstitutions of aequorin from Arabidopsis plants expressing cytosolic aequorin and HVA1-2.2 (termed "cytosolic" and "microdomain" plants, respectively) were compared. In vitro kinetics were almost identical: at least 80% of maximal aequorin activity was detected after 1 to 2 hr, and maximal activity was detected after 6 hr (Figure 5B). However, the in vivo kinetics differed. Aequorin activity continued to increase over a 24-hr time period during in vivo reconstitution in the cytosolic plants. Maximal activity was reached after 6 to 9 hr in the vacuolar microdomain plants, and by 24 hr activity had decayed to approximately half this level (Figure 5C). The similarity between in vitro reconstitutions suggests that there was no significant difference in the activities of the aequorin moieties in both types of transgenic plants. This is substantiated by the fact that no signal other than cold shock that we have investigated, for example, touch, hydrogen peroxide, and acid, produces different  $[Ca^{2+}]_{cyt}$  kinetics in the microdomain plants compared with the cytosolic plants (H. Knight, A.J. Trewavas, and M. R. Knight, unpublished results). This is in contrast to differences observed in the in vivo reconstitution, which indicated that the microdomain-reconstituted aequorin was less stable in vivo. This is most likely due to a local environment of higher free calcium concentration around the vacuolar microdomain; however, because so little is known about localized free calcium concentration in plant cells, this is not certain.

#### $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{md}$ Cold Shock Calcium Responses

Calibration of free calcium levels allowed us to compare events quantitatively in the cytosol and in the vacuolar microdomain. As shown in Figure 6, there was an obvious difference in the kinetics of the calcium response to cold shock between the microdomain and cytosolic Arabidopsis plants. In this experiment, cytosolic plants exhibited a higher peak ( $2 \pm 0.028 \mu M$ ;  $n = 5$ ) than the microdomain ( $1.4 \pm 0.061 \mu M$ ;  $n = 5$ ). In addition,  $[Ca^{2+}]_{md}$  was elevated longer than  $[Ca^{2+}]_{cyt}$ , consistent with a more prolonged elevation of free calcium concentration around the cytosolic face of the vacuolar membrane.

#### Effect of Agents Affecting Inositol 1,4,5-Trisphosphate Metabolism

Inositol 1,4,5-trisphosphate ( $IP_3$ ) is known to be able to stimulate release of  $Ca^{2+}$  from plant vacuoles (Alexandre et al.,



**Figure 6.**  $[Ca^{2+}]_{cyt}$  and  $[Ca^{2+}]_{md}$  Cold Shock Kinetics.

The trace for  $[Ca^{2+}]_{cyt}$  (CYT) is solid, and the trace for  $[Ca^{2+}]_{md}$  (MD) is dashed. Traces are averages of five individual seedlings. Vertical lines on peaks represent  $\pm$  SE.

1990; Canut et al., 1993; Allen and Sanders, 1994; Allen et al., 1995). We investigated the possibility that the more prolonged cold-induced  $Ca^{2+}$  signal seen at the tonoplast microdomain may have been due to  $IP_3$ -induced release of  $Ca^{2+}$  from the vacuole. Lithium chloride was used as a known inhibitor of the phosphatidylinositol cycle, inhibiting calcium release from intracellular pools (Berridge and Irvine, 1984). This is achieved by its inhibition of *myo*-inositol-1-phosphatase, the activity of which is inhibited by lithium in plants (Loewus and Loewus, 1982). Lithium at 25 mM inhibits cold-induced depolarization of microtubules in response to cold in spinach (Bartolo and Carter, 1992).

Pretreatment of plants in 20 mM lithium chloride for 30 min caused a decrease in the cold shock-induced  $Ca^{2+}$  peak height from  $2.2 \pm 0.051 \mu M$  ( $n = 5$ ) to  $1.6 \pm 0.2 \mu M$  ( $n = 4$ ) in cytosolic plants, as shown in Figure 7A. In plants expressing membrane-targeted aequorin, the peak was not reduced drastically ( $1.2 \pm 0.03 \mu M$  [ $n = 4$ ] compared with  $1.4 \pm 0.034 \mu M$  [ $n = 4$ ]), and lithium provoked a greater prolongation of the cold shock response (Figure 7B). Forty seconds after stimulation,  $Ca^{2+}$  levels still were substantially higher in plants treated with lithium. Pretreatment of plants with neomycin caused a shortening of the duration of the cold shock-induced  $Ca^{2+}$  peak in the cytosol (Figure 7C) but little effect on peak magnitude ( $2.1 \pm 0.053 \mu M$  [ $n = 5$ ] compared with  $2.0 \pm 0.051 \mu M$  [ $n = 5$ ]). Neomycin blocks metabolism of polyphosphoinositides (Atkinson et al., 1993) and inhibits phospholipase C, the enzyme that catalyzes the production of  $IP_3$  and diacylglycerol from phosphatidyl inositol-4,5-bisphosphate. It has been used for this purpose in plant systems (Toyada et al., 1992; Legendre et al., 1993). The effect on the microdomain plants was far more pronounced, with a significant reduction in peak height (from  $1.6 \pm 0.042 \mu M$  [ $n = 5$ ] to  $1.3 \pm 0.048 \mu M$  [ $n = 5$ ]) and a

prolonged response at considerably greater levels than controls (Figure 7D). This greater inhibition of the peak at the microdomain site suggests that  $IP_3$  might be involved in the normal calcium-cold shock response at the microdomain, acting to provoke release of calcium via tonoplast calcium channels.

### Comparison of Cold Shock $[Ca^{2+}]_{cyt}$ and Attenuation in Tobacco and Arabidopsis

As shown in Figure 8, the magnitude and duration of the cold shock  $[Ca^{2+}]_{cyt}$  response were similar in both tobacco and Arabidopsis, although slightly more prolonged in tobacco. The peak for cold shock  $[Ca^{2+}]_{cyt}$  in tobacco was  $2.2 \pm 0.045 \mu M$  ( $n = 5$ ), and in Arabidopsis it was  $2.2 \pm 0.045 \mu M$  ( $n = 5$ ). As shown in Figure 9, repetition of the cold shock stimulus 3, 10, or 30 min after an initial cold shock caused attenuation of the response in both species. In tobacco, the peak height of the response decreased from 2.2 to  $1.7 \pm 0.15 \mu M$  ( $n = 3$ ) when the stimulus was repeated after 3 min (Figure 9A). The response almost had recovered fully after 10 min, reaching  $2.0 \pm 0.042 \mu M$  ( $n = 3$ ), and after 30 min the full response was observed ( $2.3 \pm 0.034 \mu M$  [ $n = 3$ ]). In Arabidopsis, a similar reduction in response was seen after 3 min, with the peak height falling from 2.2 to  $1.7 \pm 0.2 \mu M$  ( $n = 3$ ) (Figure 9B). Recovery of the response after 10 min also was similar to that seen in tobacco ( $2.0 \pm 0.1 \mu M$  [ $n = 3$ ]), but the ability to respond to cold still was not regained fully after 30 min ( $2.0 \pm 0.1 \mu M$  [ $n = 3$ ]) (Figure 9B). These results were highly reproducible, and each peak on each graph presented is the average of three replicate traces from three individual seedlings.

### Acclimation-Associated $Ca^{2+}$ Signature

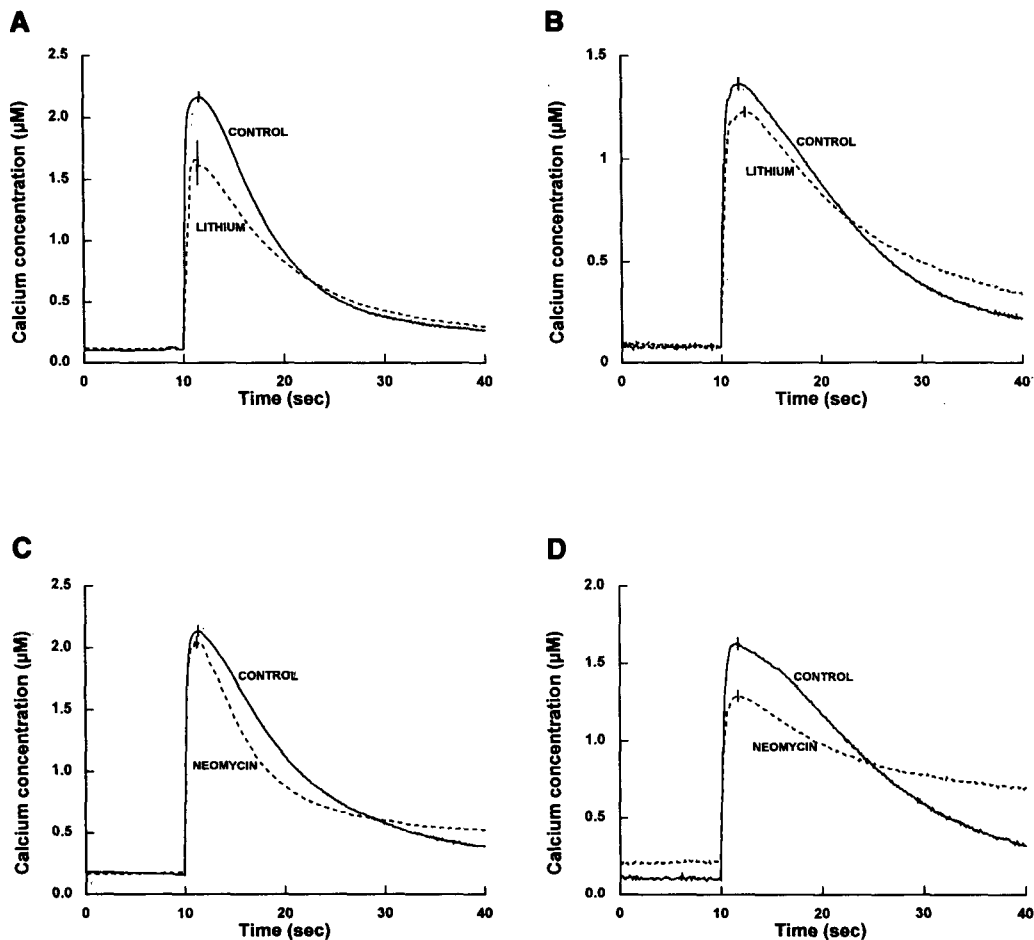
Experiments were performed to investigate the effect of cold acclimation on subsequent  $Ca^{2+}$  signaling in Arabidopsis, a chilling-resistant plant, and tobacco, a chilling-tolerant plant. Arabidopsis seedlings incubated at 4°C for a 3-hr period for 3 days exhibited altered  $[Ca^{2+}]_{cyt}$  kinetics in response to cold shock, as opposed to those continually kept at 21°C, as shown in Figure 10A. The cold shock response of the cold-pretreated plants was slightly smaller in peak height ( $2.2 \pm 0.070 \mu M$  [ $n = 7$ ] compared with  $2.4 \pm 0.032 \mu M$  [ $n = 6$ ]) and was substantially prolonged. As Figure 10B shows, no such difference was observed in similar experiments performed with tobacco.

Treatment of plants with hydrogen peroxide has been shown to mimic cold acclimation physiologically and molecularly (Prasad et al., 1994b). We were interested to determine whether this feature manifested itself as a fundamental difference in calcium signaling in Arabidopsis. The effect of hydrogen peroxide pretreatment on the subsequent calcium signature in response to cold shock also was investigated. Cytosolic plants were incubated in 10 mM hydrogen peroxide for 1 hr immediately before cold shock. Figure 11A shows the effect of hydrogen peroxide pretreatment on cold shock kinetics: cold

shock  $[Ca^{2+}]_{cyt}$  in pretreated plants was characterized by a similar peak height ( $2.3 \pm 0.027 \mu M$  [ $n = 6$ ] compared with  $2.3 \pm 0.061 \mu M$  [ $n = 6$ ]) but a slower decline in response than controls. The effect appeared similar to but less pronounced than the effect of cold pretreatment (Figure 10A).

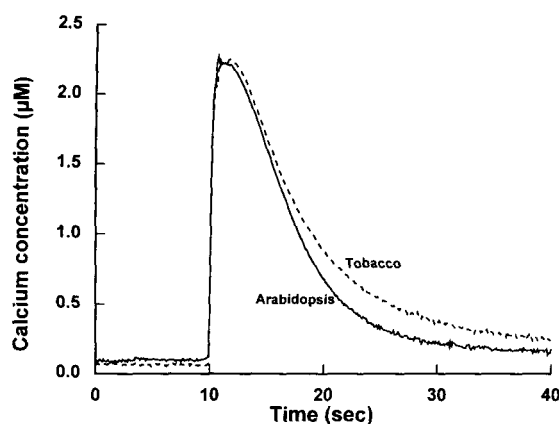
An experiment also was performed to assess whether repeated daily hydrogen peroxide treatments of plants could have an effect similar to that of cold acclimation on Arabidopsis. A more marked effect was seen with this treatment: a reduction in peak height from  $\sim 2.3 \pm 0.036 \mu M$  ( $n = 7$ ) to  $2.0 \pm 0.088 \mu M$  ( $n = 7$ ) and a more prolonged response were seen (Figure 11B), remarkably similar in appearance to the kinetics seen in cold-acclimated plants (Figure 10A). Chilling

and hydrogen peroxide treatments might be expected to act similarly because previous observations indicate these two stimuli act through a common pathway. Chilling imposes oxidative stress on maize seedlings, with hydrogen peroxide accumulation observed in the seedlings (Prasad et al., 1994a). This accumulation of hydrogen peroxide causes induction of antioxidant enzymes that scavenge hydrogen peroxide, and in this way chilling tolerance is induced (Prasad et al., 1994b). The exogenous application of hydrogen peroxide has been shown to confer cold resistance (Prasad et al., 1994a). The reciprocal also occurs, with cold acclimation demonstrated to increase tolerance of active oxygen species in cereals (Bridger et al., 1994).



**Figure 7.** Effect of Altered  $IP_3$  Metabolism on Cold Shock Calcium Kinetics in 7-Day-Old Arabidopsis Seedlings.

(A) Effect of preincubation for 30 min in 20 mM lithium on cold shock-induced  $[Ca^{2+}]_{cyt}$  elevation compared with the control. (B) Effect of preincubation for 30 min in 20 mM lithium on cold shock-induced  $[Ca^{2+}]_{md}$  elevation compared with the control. (C) Effect of preincubation for 30 min in 50  $\mu M$  neomycin on cold shock-induced  $[Ca^{2+}]_{cyt}$  elevation compared with the control. (D) Effect of preincubation for 30 min in 50  $\mu M$  neomycin on cold shock-induced  $[Ca^{2+}]_{md}$  elevation compared with the control. Traces shown are averages of four (A) and (B) or five (C) and (D) individual seedlings. For each graph, traces for inhibitor treatment are shown as dashed lines and control as solid lines. Vertical lines on peaks represent  $\pm$  SE.



**Figure 8.** Comparison of the Cold Shock  $[Ca^{2+}]_{cyt}$  Response of Tobacco and Arabidopsis Seedlings.

The trace for Arabidopsis is full-tone and the trace for tobacco is half-tone. Traces are the average of five individual seedlings.

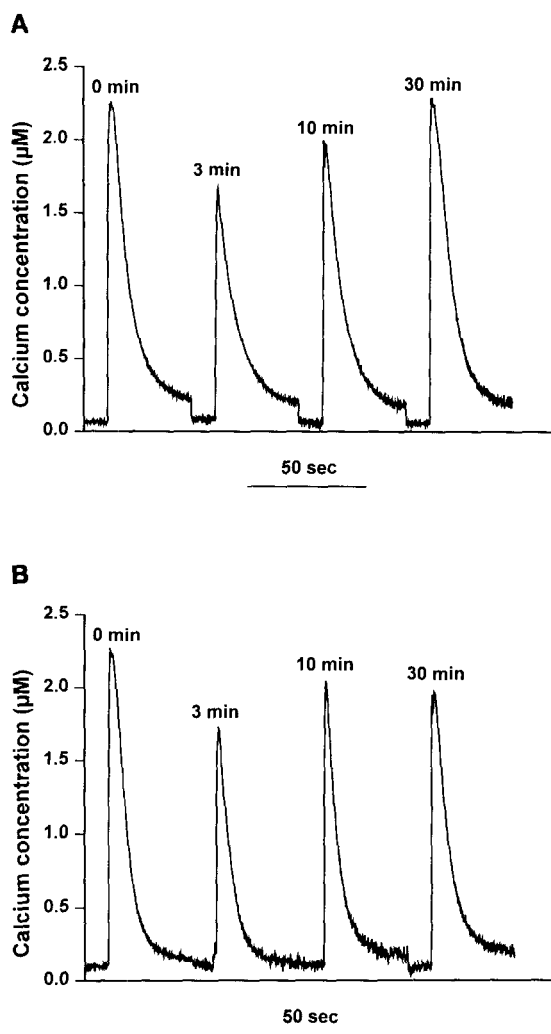
## DISCUSSION

We have demonstrated that the large increase in  $[Ca^{2+}]_{cyt}$  occurring during the cold shock response of Arabidopsis is due mainly to an influx of extracellular  $Ca^{2+}$ . Characteristically, this influx can be inhibited by preincubation in the  $Ca^{2+}$  chelator EGTA (Figure 1A) or the  $Ca^{2+}$  channel blocker lanthanum (Figure 1B). Calibrating  $[Ca^{2+}]_{cyt}$  shows that EGTA and lanthanum inhibition of the cold shock  $[Ca^{2+}]_{cyt}$  response is only partial, contrary to previous predictions (Knight et al., 1991) based on simple luminometry, which overestimates inhibition. We suggest that this partial inhibition is due to the involvement of intracellular  $Ca^{2+}$  stores. This is supported by the observation that lanthanum does not fully inhibit calcium-dependent cas gene expression in alfalfa (Monroy et al., 1993). We demonstrated that a single cold shock treatment was sufficient to cause elevated levels of *kin1* mRNA after 1 hr. Using EGTA, lanthanum, and external calcium, we demonstrated the necessity for an influx of  $Ca^{2+}$  to produce the full *kin1* response (Figure 2). These data suggest that the cold shock  $Ca^{2+}$  increase we observed is capable of eliciting downstream changes in gene expression.

We were interested to discover whether the additional component of the cold shock  $[Ca^{2+}]_{cyt}$  response was due to release of  $Ca^{2+}$  from the vacuole. We therefore targeted apoequorin to the cytosolic face of the vacuolar membrane, an area that we term the vacuolar microdomain. Lliñas et al. (1992) demonstrated  $Ca^{2+}$  levels of 200 to 300  $\mu M$  in microdomains of the cytosol on the surface of the plasma membrane during transmitter secretion in neurons, and similar microdomains of high  $Ca^{2+}$  have been demonstrated in other cellular locations (Rizzuto et al., 1993).  $Ca^{2+}$  measurement in these

specific microdomain environments can give information about organellar involvement in  $Ca^{2+}$  signaling that is not detected in general cytosolic  $Ca^{2+}$  measurements.

Fractionation of tissue from transgenic Arabidopsis showed that under the conditions we used, 57% of aequorin activity was associated with the microsomal fractions of microdomain plants (Figure 5A). This is compared with 3% detected in the microsomal fractions of cytosolic plants (Figure 5A; Knight et al., 1991). These values suggest that the  $[Ca^{2+}]_{cyt}$  events we see at the microdomain could well be underestimated. A sub-



**Figure 9.** Attenuation of Cold Shock  $[Ca^{2+}]_{cyt}$  Response.

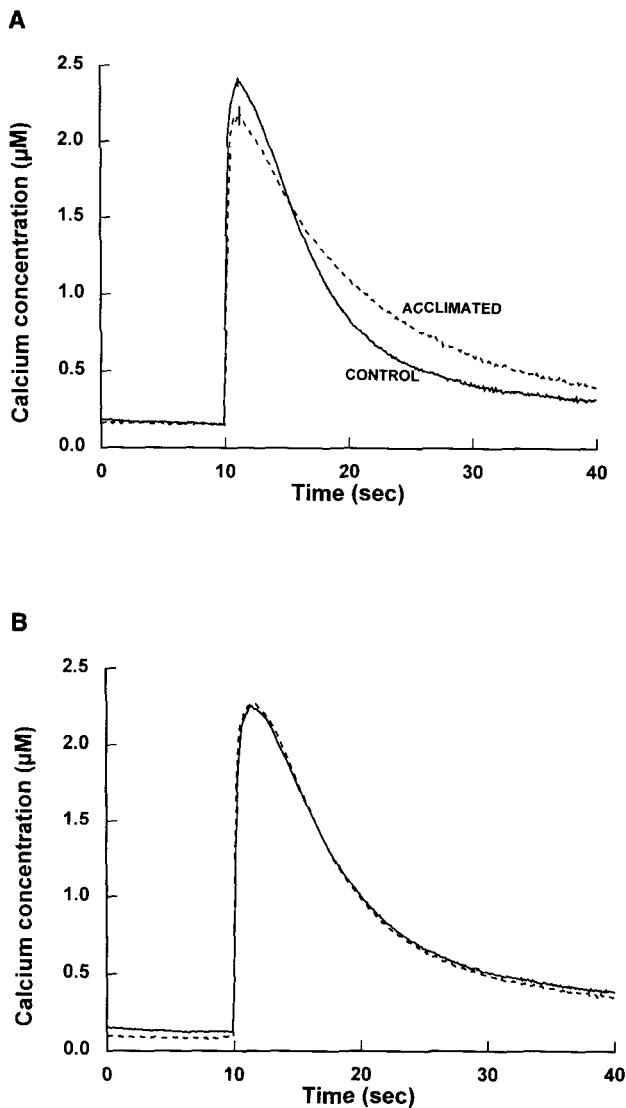
Seedlings were subjected to a cold shock once (0 min) and then to a second cold shock after 3, 10, or 30 min.

(A) Eight-day-old tobacco seedlings.

(B) Seven-day-old Arabidopsis seedlings.

Traces are all averages of three individual seedlings.





**Figure 10.** Cold Shock  $[Ca^{2+}]_{cyt}$  Response in 7-Day-Old Arabidopsis Seedlings after 3 Days of Cold Acclimation.

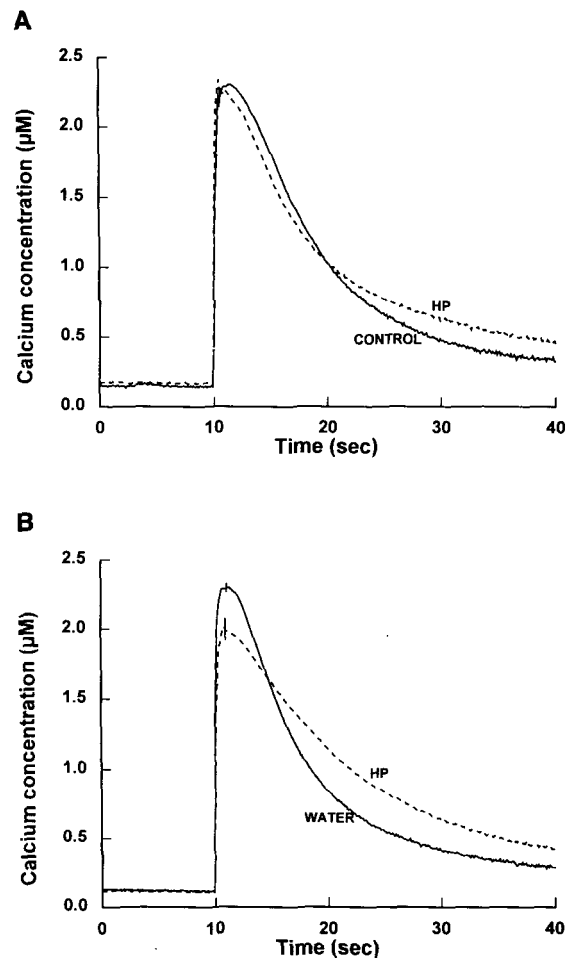
(A) A comparison of cold shock  $[Ca^{2+}]_{cyt}$  kinetics in cold-acclimated Arabidopsis (dashed line) and control Arabidopsis (solid line) is shown. Traces are averages of six (control) or seven (cold-acclimated) individual seedlings.

(B) A comparison of cold shock  $[Ca^{2+}]_{cyt}$  kinetics in cold-acclimated tobacco (dashed line) and control tobacco (solid line) is shown. Traces are averages of six individual seedlings. Vertical lines on peaks represent  $\pm$  SE.

stantial amount of aequorin activity is associated with isolated vacuoles, although it is likely that we have underestimated the true amount of aequorin associated with the tonoplast *in vivo* due to the loss of the aequorin moiety by protease activity during vacuole isolation. We assume that the aequorin associated

with the vacuole fraction is membrane bound and on the cytosolic face, because aequorin would be irreversibly inactivated by the conditions within the plant vacuole (Campbell, 1988).

Our results showed that cold shock-induced calcium changes in the vacuolar microdomain are smaller but more prolonged than those seen in the rest of the cytosol, declining more slowly in relation to their peak size. The resting level of  $Ca^{2+}$  at the vacuolar membrane was lower than we might have expected adjacent to the vacuole, which contains high  $Ca^{2+}$  levels. However, the targeted apoaequorin molecules are not necessarily close to calcium channels and therefore may not be



**Figure 11.** Effect of Hydrogen Peroxide Pretreatment on Cold Shock  $[Ca^{2+}]_{cyt}$  Response.

(A) Effect of incubation in either 10 mM hydrogen peroxide (HP; dashed line) or water control (solid line) for 1 hr before cold shock.

(B) Effect of daily spraying with either 10 mM hydrogen peroxide (dashed line) or water as a control (solid line) for 3 successive days before cold shock.

Traces are averages of six (A) or seven (B) individual seedlings. Vertical lines on peaks represent  $\pm$  SE.

expected to report any high levels of calcium that would be expected to occur locally around these channels.

Therefore, our data suggest that cold shock  $[Ca^{2+}]_{cyt}$  responses involve both an influx of external  $Ca^{2+}$  and a  $Ca^{2+}$  signaling event at the vacuolar membrane. The slower decline in  $Ca^{2+}$  seen in the microdomain in comparison with the cytosol is consistent with either uptake of  $Ca^{2+}$  into the vacuole or a more prolonged release of  $Ca^{2+}$  from the vacuole, continuing after  $Ca^{2+}$  influx across the plasma membrane has finished.

There are clearly three possibilities for the vacuolar release of  $Ca^{2+}$  following cold shock: a direct effect of cold on the vacuole and its calcium channel activity; a calcium-induced calcium release, perhaps via a slow vacuolar channel (Ward and Schroeder, 1994) triggered by the influx of extracellular calcium; or an  $IP_3$ -mediated effect of the influx of extracellular calcium. We cannot discount a direct effect of cold on the vacuole. We do not have any data to support the hypothesis of calcium-induced calcium release. Indeed, causing an influx of extracellular calcium to produce  $[Ca^{2+}]_{cyt}$  of up to 1  $\mu M$  by addition of 100 mM external calcium produced no detectable calcium-induced calcium release (H. Knight, A.J. Trewavas, and M.R. Knight, unpublished results). In addition, if we assume that the long period of preincubation in high concentrations of EGTA completely inhibits the  $Ca^{2+}$  influx, the remaining  $Ca^{2+}$  elevation must occur independently of this influx.

Our studies with the inhibitors neomycin and lithium suggest there may be a role for  $IP_3$ -mediated calcium release from the vacuole. Neomycin blocks metabolism of phosphoinositides, thus reducing  $IP_3$  synthesis. Neomycin reduces the duration of the  $Ca^{2+}$  spike in the cytosol. This may be due to a decrease in activity of an  $IP_3$ -mediated  $Ca^{2+}$  release and, if so, would indicate that an  $IP_3$ -mediated  $Ca^{2+}$  release may contribute to the full elevation in  $[Ca^{2+}]_{cyt}$  in response to cold shock. Neomycin has a greater inhibitory effect on  $Ca^{2+}$  elevation in the vacuolar microdomain compared with the cytosol in general, and this suggests an  $IP_3$ -mediated event at this location. After cold shock, subsequent resting levels of  $Ca^{2+}$  at the vacuolar microdomain were higher in neomycin-pretreated plants than in controls. This could be due to a slower breakdown of  $IP_3$  due to neomycin binding to it or due to  $Ca^{2+}$  mobilization at the vacuolar membrane by neomycin, which acts in a similar manner on intracellular  $Ca^{2+}$  stores in animal cells (Nakashima et al., 1987).

Lithium, which inhibits the cycling of inositol phosphates, reduced the magnitude of the  $Ca^{2+}$  peak height in both cytosolic and vacuolar microdomain plants. This may be due to a reduced availability of  $IP_3$  at the start of the response and may be because part of the initial elevation of  $Ca^{2+}$  is due to  $IP_3$ -mediated  $Ca^{2+}$  release. Lithium inhibition of inositol-1,4-bisphosphate and  $IP_3$  degradation (Martinoia et al., 1993) would increase the time over which any  $IP_3$  present can exert an effect on  $Ca^{2+}$  stores. The prolonged response seen in vacuolar membrane-targeted plants may be due to a prolonged elevation of  $IP_3$  levels.

The comparison of cold shock responses in tobacco (cold sensitive) and Arabidopsis (cold tolerant) showed that the  $[Ca^{2+}]_{cyt}$  kinetics in both are similar in magnitude and duration, although the tobacco response is slightly longer lived (Figure 8). The similarity between these two species suggests that under normal circumstances, calcium signaling perhaps does not play a role in the differences observed between the physiology of these two species in response to low temperature. To determine whether this becomes an issue during acclimation and prolonged cold treatment, we investigated calcium signaling during multiple cold stimulation and cold acclimation.

We observed that tobacco was able to recover its ability to respond fully to cold shock 30 min after an initial cold shock, whereas Arabidopsis was not (Figure 9). We speculated that this longer recovery time in Arabidopsis may form part of a "cold memory" and that cold-sensitive tobacco plants were less able to retain information on previous cold treatment. This implies that Arabidopsis had a more long-lived and significant cold memory.

To investigate whether the memory manifested itself as a change in  $Ca^{2+}$  signaling, we compared the  $[Ca^{2+}]_{cyt}$  kinetics in cold-acclimated and nonacclimated Arabidopsis plants. Calcium has been implicated in the transduction of cold acclimation (Monroy et al., 1993; Monroy and Dhindsa, 1995). Cold acclimation is the process whereby chilling to noninjurious above-zero temperatures induces tolerance of subsequent freezing temperatures and has been observed in a number of species (Guy, 1990; Jennings and Saltveit, 1994). Several methods have been suggested for cold acclimation, including the restructuring of subcellular membranes to avoid freezing damage (Thompson, 1986). Our results show that short-term (Figure 9B) and long-term (Figure 10A) cold acclimation altered the cold shock  $[Ca^{2+}]_{cyt}$  signature in Arabidopsis. Cold acclimation produced a  $[Ca^{2+}]_{cyt}$  signature with a reduced peak height and a prolonged profile (Figures 9B and 10A).

Hydrogen peroxide has been shown to induce chilling stress and increases expression of chilling acclimation-responsive (CAR) genes (Prasad et al., 1994a). One of these genes encodes a catalase that scavenges superoxides, thereby preventing them from accumulating and causing oxidative damage to the plant. It also has been shown that chilling induces an increase in respiration via the alternative cyanide-resistant pathway (Moynihan et al., 1995). This may have a beneficial role in reducing superoxide levels and thus reducing oxidative damage to the mitochondria (Prasad et al., 1994b). Because hydrogen peroxide can mimic cold acclimation and endow cold tolerance (Prasad et al., 1994a), we were interested in determining whether hydrogen peroxide altered the cold shock  $[Ca^{2+}]_{cyt}$  signature in the same way as acclimation. We found that both short-term (Figure 11A) and long-term (Figure 11B) treatment of Arabidopsis with hydrogen peroxide produced an altered  $[Ca^{2+}]_{cyt}$  signature similar to the one associated with cold acclimation (Figure 10A). Our results with cold acclimation and hydrogen peroxide pretreatment suggest that these two treatments act through a similar pathway.

We have shown that treatments acclimatizing plants to low temperature, that is, hydrogen peroxide and prolonged cold treatment, are associated with a modified, characteristic calcium signature. It seems possible, therefore, that cold acclimation involves the modulation of plant calcium signaling to prepare the plant for future episodes of low temperature. Because inhibition of  $\text{Ca}^{2+}$  influx fully inhibits cold acclimation but not *cas* gene expression (Monroy et al., 1993), two possibilities exist for the  $\text{Ca}^{2+}$  regulation of cold acclimation. Either cold-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  needs to reach a certain threshold value to activate acclimation or acclimation requires  $\text{Ca}^{2+}$  influx through the plasma membrane specifically. To resolve these two possibilities, we are investigating the calcium signaling around the plasma membrane during cold acclimation by using plants with aequorin targeted to the plasma membrane.

## METHODS

### Plant and Bacterial Material

Transgenic *Arabidopsis thaliana* (a kind gift from J. Braam and D.H. Polisensky, Rice University, Houston, TX) and tobacco (*Nicotiana plumbaginifolia* line MAQ2.4; Knight et al., 1991) plants both expressing cytosolic apoequorin were used for cytosolic free calcium concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) measurements. Plants expressing the apoequorin-pyrophosphatase fusion protein (Arabidopsis line HVA1-2.2) were produced as detailed below. *Escherichia coli* JM101 and XL-1 Blue were used as hosts for all recombinant DNA manipulations. *Agrobacterium tumefaciens* C58C1 and Arabidopsis ecotype C24 were used for plant genetic transformation. Seedlings were grown on full-strength Murashige and Skoog medium (Murashige and Skoog, 1962), 0.8% agar at 21°C (Arabidopsis) or 25°C (tobacco), with a 16-hr photoperiod, and were used when 6 to 7 days old (Arabidopsis) or 7 to 10 days old (tobacco).

### Chemicals

All enzymes used for DNA manipulations were purchased from Life Technologies (Paisley, UK), as was the TRIzol reagent. DNA isolation kits were from Promega (Southampton, UK) and Qiagen (Dorking, UK), the agar was from Unipath (Basingstoke, UK), and all plant tissue culture reagents and other chemicals were from Sigma. Macerozyme and cellulase used for production of protoplasts were from Serva (Wokingham, UK). Coelenterazine was purchased from Molecular Probes (Eugene, OR). Oligonucleotide primers were prepared by using a DNA synthesizer (model 392A; Applied Biosystems, Warrington, UK), and their sequences (5' to 3') were as follows: C206, CCGTCGACCGAAGTACTTGAAAGGATAC (3' antisense primer for pyrophosphatase sequence, including Sall site); C207, GGTCTAGATATACGGAGGAGAGAAGATG (5' sense primer for pyrophosphatase sequence, including XbaI site); A993, CCGTCGACAAATGACCGAGCAACAATACTCA (5' sense primer for apoequorin sequence, including Sall site); A175F, GGCTGCAGCGAGGTTTCTTAGGGGACA (3' antisense primer for apoequorin cDNA, including PstI site); *kin1* forward, ATGTCAGAGACCAACAAGAA (5' sense primer for the *kin1* sequence, beginning at the start codon); and *kin1* reverse, GAATATAAGTTGGCTCGTC (3' antisense primer for the *kin1* sequence).

### Production of a Chimeric Construct Encoding a Proton Pyrophosphatase-Apoequorin Fusion Protein and Genetic Transformation of Arabidopsis

The construct HVA1 was designed to have the coding sequence from a full-length cDNA clone for a vacuolar pyrophosphatase protein attached to the 5' end of the coding sequence from a cDNA clone for apoequorin, thus preserving the stability of the aequorin moiety (Watkins and Campbell, 1993). The Arabidopsis vacuolar membrane pyrophosphatase-energized proton pump was chosen because it is a single-subunit protein. The protein as predicted from cDNA sequence (Sarafian et al., 1992) has 770 amino acids. The sequences of this cDNA and the aequorin cDNA were modified by using polymerase chain reaction (PCR) to add restriction endonuclease sites to the 3' and 5' termini of both sequences and thus facilitate cloning into the vector pDH51 (Pietrzak et al., 1986). The full coding sequence for both proteins was used.

The unmodified apoequorin cDNA used as the template for PCR was the PstI fragment of plasmid pAEQ1 (Cormier et al., 1989), and the EcoRI fragment of plasmid pAVP-3 (a kind gift from P. Rea, University of Pennsylvania, Philadelphia; Sarafian et al., 1992) was used as template for the pyrophosphatase PCR. A Sall site was introduced at the 5' end of the aequorin cDNA by using oligonucleotide A993, and a PstI site was introduced at the 3' end by using oligonucleotide 175F. The pyrophosphatase cDNA was modified by introducing an XbaI site to the 5' end by using oligonucleotide C207 and a Sall site to the 3' end by using oligonucleotide C206. PCR products were digested with Sall and XbaI or PstI and successively cloned into pDH51. This vector contains a cauliflower mosaic virus 35S transcription promoter and terminator (Pietrzak et al., 1986). The whole chimeric gene was transferred to the binary vector pBIN19 (Bevan, 1984) to produce the plasmid pHVA1. The plasmid was purified from *E. coli* and then used to transform *Agrobacterium* by a freeze-thaw method (Holsters et al., 1978). Arabidopsis was transformed using a modified version of the Valvekens protocol for root transformation (Valvekens et al., 1988; Balcells, 1992).

### Subcellular Fractionation of Plant Tissue

For microsomal fraction preparations, 10 g of tissue, from 3- to 4-week-old plants, was used per transformed line. The tissue was chopped up roughly with scissors and homogenized with a mortar and pestle on ice in 20 mL of modified reconstitution medium (10 mM Tris-HCl, pH 7.45, 5 mM EDTA, 5 mM  $\beta$ -mercaptoethanol, 0.1% gelatin, 500 mM mannitol). The homogenate was filtered through two layers of muslin, and the filtrate was spun at 13,000g in a bench centrifuge (Beckman Instruments) for 30 min at 4°C to remove cell debris. The supernatant was then spun at 80,000g in a centrifuge (model L8-M; Beckman Instruments) at 4°C for 50 min. The supernatant was removed, and an aliquot was taken. This supernatant contained the cytosolic component of the cells, whereas the pellet contained the microsomal membranes and vesicles. The pellet was resuspended in 1 mL of normal reconstitution medium (as described above, but containing 0.5 M NaCl and without mannitol) and diluted 9:1 with reconstitution medium containing 500 mM mannitol. Aliquots (100  $\mu$ L) of the pellet resuspension and the supernatants were taken, and coelenterazine was added to a concentration of 1  $\mu$ M and allowed to reconstitute for 1.5 hr.

Vacuolar localization was performed by preparing protoplasts from 1.5 g of 4-week-old plant tissue by standard methods, using macerozyme and cellulase and purification on a sucrose cushion (Wei and Lindsey, 1992). Protoplasts collected were lysed by using a Ficoll-

containing buffer to release vacuoles according to the method of Höfte and Chrispeels (1992) modified for Arabidopsis (Hauser, 1992). The vacuoles were purified on a Ficoll gradient, and the relative numbers of vacuoles in the unfractionated lysed protoplast suspension and in the final purified vacuolar fraction were determined by assaying for activity of the vacuolar enzyme acid phosphatase (Höfte and Chrispeels, 1992). The enzyme activity was assayed at room temperature by using *p*-nitrophenyl phosphate (1 mg/mL) as substrate, and the reaction was stopped with excess sodium carbonate in a microtiter plate. The amount of product was determined by measuring absorbance at 415 nm with a microtiter plate reader. The relative number of vacuoles was used to relate the number of vacuoles in the vacuolar fraction to the number of vacuoles in the protoplast fraction. Both fractions were assayed for aequorin activity by reconstituting aequorin *in vitro*.

### In Vitro and in Vivo Reconstitution of Aequorin

Reconstitution of aequorin was performed *in vitro* and *in vivo* essentially as described previously (Knight et al., 1991). An *in vitro* time course of reconstitution was performed by homogenizing three plants in 0.5 mL of reconstitution buffer (0.5 M sodium chloride, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM  $\beta$ -mercaptoethanol, 0.1% gelatin), spinning the tubes for 10 min in a microcentrifuge, and taking 100  $\mu$ L of supernatant, to which coelenterazine was added to a concentration of 1  $\mu$ M. Reconstitution was allowed to occur for 1.5 hr in darkness, after which 10  $\mu$ L of the reconstituted aequorin mixture was added to 0.5 mL of 200 mM Tris, 5  $\mu$ M EDTA, pH 7, and discharged in the luminometer by the addition of an equal volume of 50 mM  $\text{CaCl}_2$  after 0.5, 1, 3, 6, 9, and 24 hr of reconstitution. Aequorin was reconstituted *in vivo* by floating seedlings on water containing 2.5  $\mu$ M coelenterazine overnight in the dark at room temperature. The time course of *in vivo* reconstitution was performed by discharging the reconstituted aequorin in intact seedlings by the addition of 1 M  $\text{CaCl}_2$ , 10% ethanol after 0.5, 1, 3, 6, 9, and 24 hr.

### Cold Shock Calcium Measurements

Experiments were performed by placing individual seedlings in a transparent plastic cuvette without liquid. The cuvette was placed inside a digital chemiluminometer. This consisted of a 9829A photomultiplier tube with a 1.5-kV potential from a PM28B high-voltage supply cooled to  $-25^\circ\text{C}$  by using a FACT50 air-cooled thermoelectric housing and an AD2 amplifier/discriminator (all from Thorn EMI, Ruislip, UK) connected as previously described (Campbell, 1988). Output from the amplifier/discriminator was channeled via a CT1 computer counter board (Thorn EMI) to produce numerical output, which was stored on a personal computer. Luminescence counts were recorded every 0.1 or 1 sec. After 10 sec of counting, 1 mL of ice-cold water was injected into the cuvette via a light-tight 2-mL syringe inserted into a light-tight port in the luminometer sample housing. At the end of each experiment, the remaining aequorin was discharged by the addition of 1 mL of 2 M  $\text{CaCl}_2$  and 20% ethanol. Inhibitor experiments were performed by placing reconstituted seedlings in the appropriate inhibitor or water for a fixed period of time, after which seedlings were removed from the inhibitor and placed in a cuvette. Cold shock-induced luminescence was measured immediately as given above.

Attenuation was examined by measuring cold shock luminescence in both tobacco and Arabidopsis. Subsequent seedlings were subjected to one cold shock without recording luminescence counts, and

after 3, 10, or 30 min, they were subjected to a second cold shock, during which changes in luminescence were measured. Aequorin was discharged as usual at the end of the experiment. In this way, aequorin calibration was not subjected to alteration by any further reconstitution that may have occurred between the first and second cold shocks.

To investigate acclimation, agar plates with 3-day-old Arabidopsis and 9-day-old tobacco seedlings expressing cytosolic aequorin were placed at either 4 or  $21^\circ\text{C}$  in darkness for 3 hr on 3 successive days. On the third day, the seedlings were returned to their normal growth conditions for 4 hr, after which they were removed from the plates and reconstituted in coelenterazine overnight. On the following day, cold shock measurements were made in the usual way. No noticeable differences in growth and development were observed between treated and untreated plants.

For hydrogen peroxide pretreatment, 7-day-old Arabidopsis seedlings expressing cytosolic aequorin were floated on a solution of 10 mM hydrogen peroxide for 1 hr immediately before measurement of cold shock. In other experiments, 3-day-old seedlings on agar plates were sprayed with either hydrogen peroxide (10 mM) or water once a day for the following 3 days by using a manual atomizer. Seven hours after the final spraying, the seedlings were removed from the agar and reconstituted overnight as normal.

### Calibration of Calcium Measurements

A double logarithmic relationship exists between the concentration of free calcium present in cells containing reconstituted aequorin and the proportion of total remaining aequorin present in the cell that is discharged at any point in time (Blinks et al., 1978). Calibrations were performed by estimating the amount of aequorin remaining at the end of experiment by discharging all remaining aequorin in 1 M  $\text{CaCl}_2$ , 10% ethanol. Calibrations were performed by using a calibration equation derived empirically from measurements made with the aequorin sequence encoded by the specific PCR product we used to produce the chimeric constructs for plant transformations (data not shown) and at the temperature at which our experiments were performed. This provides a more accurate calibration than the standard normally used, which was determined by using a mixture of aequorin isoforms at  $37^\circ\text{C}$  (Cobbold and Rink, 1987) and has been used previously to calibrate plant  $[\text{Ca}^{2+}]_{\text{cyt}}$  (Haley et al., 1995). The calibration equation is as follows, where  $k$  is a rate constant equal to luminescence counts per second divided by total remaining counts:  $\text{pCa} = 0.332588(-\log k) + 5.5593$ .

### Measurement of Cold-Induced *kin1* Gene Expression by Using Reverse Transcription PCR

Primers for the specific amplification of the *kin1* transcript were designed. The reverse primer sequence was designed to be incorrect for amplification of the *kin2* transcript. When amplifying from cDNA, these oligonucleotides produce a product with a predicted size of 342 bp. The degree of accumulation of this transcript after cold shock was used as a measure of an end response to cold shock.

Approximately 20 to 25 mg of 7-day-old Arabidopsis seedlings expressing cytosolic aequorin was placed in water or inhibitor solutions (10 mM lanthanum chloride, 20 M EGTA, 100 mM lithium chloride, and 200  $\mu$ M neomycin sulfate) for 1 hr. Seedlings were cold shock treated as they were for calcium measurements. Water at ambient tempera-

ture was used for controls. Another treatment involved adding 100 mM calcium chloride instead of cold water.

Total RNA was prepared from seedling tissue 1 hr after treatments by using TRIzol reagent, and cDNA was synthesized. For each reaction, 1 µg of total RNA was used, and 1 µL of oligo(dT) (500 µg/mL) was added in a total volume of 12 µL. The mixture was heated to 70°C for 10 min and quick chilled on ice to denature the RNA; 4 µL of first-strand buffer, 2 µL of 0.1 M DTT, 1 µL of 10 mM deoxynucleotide triphosphates, and 1 µL (200 units) of Superscript II (RNAse H<sup>-</sup> reverse transcriptase) were then added. cDNA synthesis was performed by incubating at 42°C for 50 min, and the enzyme was inactivated at 70°C for 15 min. The cDNA was diluted 1:100, and 10 µL was used for PCR with the specific *kin1* primers (see above). The cDNA was amplified under the following conditions: 94°C for 5 min, 60°C for 5 min, and 72°C for 5 min; followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and ending with 15 min at 72°C. Control PCR was performed with 10 µL of a 1:10,000 dilution of the cDNA and previously made primers to the aequorin gene (Knight et al., 1991) under the same conditions. Equal loading of each *kin1*-amplified sequence was determined by loading equal volumes of the control aequorin PCR.

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